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PRINCIPAL INVESTIGATOR: Arnaud Monvoisin, Ph.D.

CONTRACTING ORGANIZATION: The University of California, Los Angeles
Los Angeles, California 90024-1406

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Arnaud Monvoisin, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of California, Los Angeles
Los Angeles, California 90024-1406

E-Mail: arnaud@ucla.edu

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13. ABSTRACT (Maximum 200 Words)

Vascular endothelial growth factor is a critical mediator of angiogenesis. Levels of this cytokine are under exquisite transcriptional and translational control and alterations in can have devastating effects in development. In this study, we demonstrated that VEGF is also regulated extracellularly by matrix metalloproteases. Specifically, MMP3 is capable to cleave the three main splice forms of this growth factor releasing bioactive fragments with equal ability to induce VEGFR2 phosphorylation in vitro. We have mapped the regions of MMP3 processing and generated recombinant forms that mimic MMP3-cleaved VEGF and MMP3-resistant VEGF to explore the biological relevance of this processing event. We found that cleavage of VEGF impacts vessel size, vascular density, and vascular branching. MMP-cleaved VEGF is less able to support tumor growth than MMP-resistant VEGF. MMP-cleaved VEGF induces capillary hyperplasia in existent vessels, but induces a poor angiogenic response. In contrast, MMP-resistant VEGF supports robust growth of thin vessels with frequent branching points. In addition, this form is conducive to metastatic events in cell lines that normally do not metastasize. These findings reveal a novel mode of regulation of VEGF that impacts all spliced forms and have modulatory effects in tumor growth and vessel morphogenesis.

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Introduction

Matrix metalloproteases (MMPs) play a significant role in remodeling the extracellular environment, enable migration and establishment of new capillary beds. This fellowship aims to gain a deeper understanding of the interplay between these molecules and vascular endothelial growth factor (VEGF). We have investigated the potential of matrix metalloproteases to release VEGF from extracellular stores. During this process we found that VEGF can be cleaved intramolecularly by several MMPs. MMP3 in particular is able to release bioactive VEGF fragments. This carries significance to tumor angiogenesis since VEGF is frequently secreted and bound to heparin-sulfate proteoglycans and to other proteins in the extracellular matrix. During the course of this fellowship, we have mapped the sites cleavage and generated proteins that were able to mimic both the cleaved form, as well as a form unable to be cleaved. Recombinant proteins from the soluble and MMP3-resistant VEGF forms were equally able to phosphorylate VEGFR2 *in vitro*. These data has been concluded and has been described in detail in the previous progress report.

During this last year, we have concluded the xenographs assays using tumor cells transfected with the different forms of VEGF. Interestingly and contrary to our expectations, the soluble form generated small tumors as compared with the tumors generated by the MMP3-resistant form of VEGF. Circulating levels of VEGF also contrasted the size of the tumors, thus the soluble form showed 4-5-fold excess of circulating VEGF when compared to the MMP3-resistant form. Vascular branching was notably increased in the MMP-resistant form and reminiscent of the effects of VEGF189. These findings reveal that levels of MMP can modulate the biological effects of VEGF in tumors with significant consequences to tumor progression.

In addition, parallel efforts were initiated on a project that explores the contribution of endothelial progenitor cells (EPCs) to the tumor vasculature. The laboratory has generated transgenic animals that express Cre-recombinase directed by the vascular endothelial cadherin promoter (VE-Cadherin).

As expected, VE-Cad/Cre transgenic mice express Cre activity in fully differentiated endothelial cells. However our analysis also revealed expression in bone marrow-derived EPCs as assayed using a variety of *in vivo* and *in vitro* methods. We found that these cells become incorporated in the vasculature of tumors during xenograft experiments.

Although this research was not a direct component of the fellowship application initially submitted, it is directly pertinent to the question of therapeutic management of the vasculature in tumors. The progress in this project is presented within the body of this report. Please note that the development of this second project was in addition to the contract of this fellowship and not instead of the last aim in this fellowship. As can be seen from this report all of the aims have been addressed and we are on the verge of completing Aim 3.

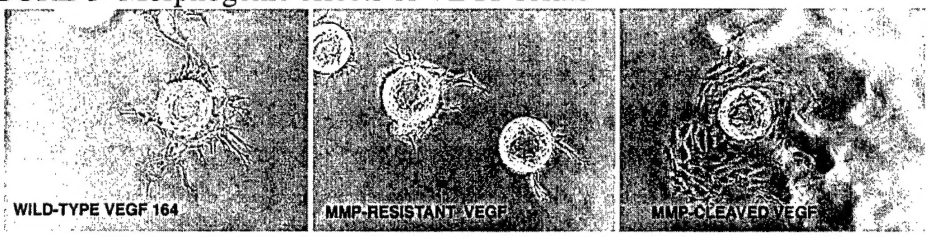
Body

Task 1 and most of task 2 were completed as per prior report.
This year we have completed the following:

Task2 - To determine the relevance of the released peptides to signal tranduction –

In the previous report, we showed that the MMP-cleaved VEGF, as well as, the uncleavable form were both able to phosphorylate VEGFR2 in an equivalent manner (time and level of phosphorylation). We have taken those experiments further and demonstrated that cleaved and uncleaved VEGF induced different cellular responses using three-dimensional assays of angiogenesis. In response to MMP-resistant VEGF, cells organize solid cord-like structures, while the cleaved VEGF induces proliferation of cells in sheets. The experiments have been done using identical molar ratios for each protein. Controls, such as endothelial cells that lack VEGF receptors do not leave the collagen-coated beads, indicating that the effect is specific. In more recent experiments, we found that MMP-cleaved VEGF activate Akt, while MMP-resistant VEGF leads to activation of p38 and very poor activation of Akt. These are very preliminary, but exciting results because they have the potential to explore the differences between the proliferative and the morphogenetic signals known to be elicited by VEGF. While these signaling events have not been proposed in the original application (we could not predict that our research would take this direction) we will embrace these experiments and follow up with more detail signaling experiments to address the differences between matrix-bound and soluble VEGF.

FIGURE 1- Morphogenic effects of VEGF forms



Task 3 – To ascertain the contribution of the MMP3-VEGF axis towards prostate cancer progression and establishment of metastatic disease.

We generated stable human cell lines that express wild-type VEGF 165, a mutant form that resemble the fragment of VEGF after cleavage by MMP3 (named VEGF 113) and a mutant form of VEGF unable to be cleaved by this protease (delta 109-118).

Levels of VEGF were evaluated in Western blots (Figure 1) and subsequently cells were injected into nude mice to ascertain tumor growth kinetics (Figure 2). As shown in the examples provided in Figure 2, the tumors from VEGF113 showed the smallest size, in contrast, tumors from uncleavable VEGF showed the largest tumors. Interestingly, levels of circulating VEGF were highest in the VEGF 113 (reaching 700pg/ml) and contrasting 200-400pg/ml range from wild-type VEGF and delta 109-118, indicating that VEGF amounts are not necessarily reflective of the angiogenic response. We are currently

expanding the number of xenograft assays performed to ensure reproducibility and we are also examining tumor morphology and vascular patterning, this evaluation will mark the conclusion of the project as designed.

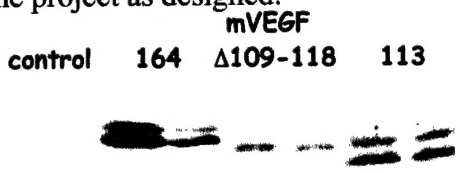


FIGURE 2

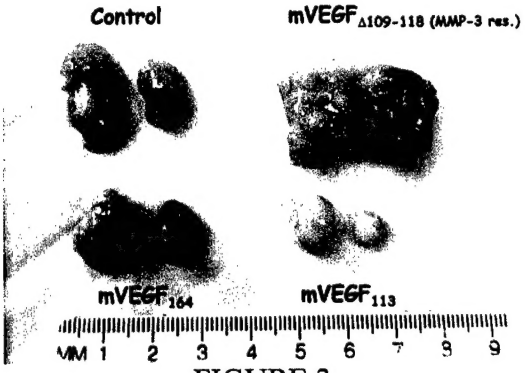


FIGURE 3

We are currently expanding this experiment (xenograft assay) to include two additional cell lines. This will put to test the reproducibility of these findings. The xenografts of these cell lines will be concluded in three more weeks. We will also modulate the levels of MMPs with adenoviral vectors as proposed in the application. Note that this is what has been presented in the contract and will be concluded. In addition (not instead) we have also embarked on a parallel very exciting project (as presented below).

Additional Project:

Using a transgenic animal that expresses the recombinase Cre under the regulation of the endothelial-specific VE-Cadherin promoter (VE-Cad), and a reporter mouse line (called ROSA26) we have identified a population of cells within the bone marrow of adult mice able to selectively engraft as into neovessels and differentiate into endothelial cells in tumor *in vivo* (figure 4).

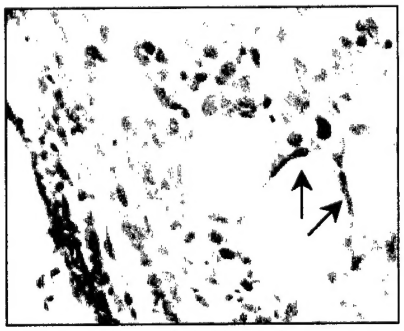


FIGURE 4

Key Research Accomplishment

Task 1 – Concluded – it has been described in the previous report.
Task 2 – Concluded – Most of it has been summarized in the previous report, some of the key findings are also described in this report.

- We found that while VEGFR2 levels are equivalent in cleaved and MMP-resistant forms of VEGF, cellular angiogenic responses are different. Cleaved VEGF elicits growth of endothelial cells as sheets, while MMP-resistant growth factor induces the growth of endothelial cells in cords and morphogenesis of capillaries. The implications of this findings are significant as they could explain the mechanisms by which VEGF mediates proliferative versus differentiation signals.

Task 3 – To ascertain the contribution of the MMP3-VEGF axis towards prostate cancer progression and establishment of metastatic disease. This task in near completion.

- We have been able to generate cell lines that express different types of VEGF (wild-type, cleaved, and MMP-resistant)
- These cell lines were injected in the flank of nude mice and evaluated in xenograft assays. A cohort of five mice per cell line showed that the MMP-resistant form induces the growth of thin and frequently branched capillaries. In contrast, cleaved VEGF results in few and enlarged tumor vessels.

Reportable Outcomes

Research

Abstracts

- Monvoisin A., Alva J., Porter J., Lane T., Iruela-Arispe M.L. 2003. *Temporally-controlled specific recombination in the endothelium in vivo. VE-cadherin/Cre mice, a new model for endothelial genes analysis.*
  Retreat of the Department of Molecular, Cell and Developmental Biology – UCLA, Lake Arrowhead, California.

- Monvoisin A., Alva J., Porter J., Lane T., Iruela-Arispe M.L. 2004. *Temporally-controlled specific recombination in the endothelium in vivo. VE-cadherin/Cre mice, a new model for endothelial genes analysis.* Cardiovasc Pathol 13 (Iss 3, Suppl 1):37-38. Abstract.
  XIII International Vascular Biology Meeting, Toronto, Canada.

Temporally-controlled specific recombination in the endothelium *in vivo*.

VE-Cadherin/Cre mice, a new model for endothelial genes analysis

Monvoisin A.[1], Alva J.[1], Porter J.[1], Lane T.[2], Iruela-Arispe M.L[1]. *[1]Department of Molecular Cellular and Developmental Biology and Molecular Biology Institute, UCLA; [2]Department of Obstetrics and Gynecology, UCLA, Los Angeles, CA 90095, USA.*

The generation of conditional mouse mutants is a powerful tool to analyze the function of a given gene product in a defined population of cells. To induce mutations in a temporally controlled manner in the endothelium, we established and characterized transgenic mice expressing a tamoxifen-inducible Cre recombinase under the control of 2.4 kb of the VE-cadherin promoter (VE-cad/Cre mice). The sequence and the activity of the promoter have been detailed previously (Gory S. et al, *Blood* 1999). The construct also contained a mutated version of estrogen receptor that allows the translocation of the Cre recombinase to the nucleus after binding to tamoxifen. However this construct is not responsive to endogenous estradiol.

Transgenic mice were generated by injection of the construct into pronuclei of fertilized eggs. We obtained 9 transgenic founder mice. From these, only 3 transmitted the transgene to the progeny. These 3 mouse lines were crossed to the reporter strain *ROSA26* in which a floxed-STOP cassette has been introduced upstream of a bacterial beta-galactosidase (Lac Z) reporter gene. Activity of Cre recombinase in the offspring (age 10-12 weeks) was examined after intraperitoneal injection of 2 mg of tamoxifen for 3 consecutive weeks. The day after the last injection, mice were euthanized, perfused and the abdominal wall muscle dissected and tested for beta-galactosidase activity. We observed activity specifically in the endothelium with marked differences in efficiency from mice to mice.

Cre recombinase activity was also detected in the vasculature of several organs, however their frequency varied significantly even in the same transgenic line. Interestingly, consistent recombination was detected in neovessels that grew in response to an inductive stimulus. In particular, vessels from cornea pocket assays performed during tamoxifen induction were lined by beta-galactosidase positive endothelial cells. Together these results reveal sporatic Cre recombinase activity in quiescent vessels and more consistent expression in vessels formed after pro-angiogenic stimulation.

These VE-Cad/Cre mice will allow us to inactivate specifically endothelial genes and to determine their roles particularly in processes that require neovascularization such as tumor growth.

Products

Cell Lines, Tissues or Serum Repositories Developed

- Generated stable human cell lines expressing wild-type VEGF165 ; amutant VEGF that resembles the fragment of VEGF generated by MMP-3 cleavage (VEGF113); or a mutant VEGF that cannot be cleaved by MMP-3 (delta 109-118).

Animal Model

- Generated a transgenic mice expressing Cre in mature endothelial cells and endothelial progenitor cells; this transgenic mouse line will allow us to induce mutations specifically in the endothelium.

Conclusions

Regarding the MMP3-VEGF project

The project is nearly completion and we will be submitting a manuscript on this work within the next couple of months.

As shown above, we have been able to demonstrate that MMP3 releases bioactive VEGF fragments that are capable to modulate angiogenesis in tumors. Interestingly, uncleaved VEGF induces a more drastic angiogenic response than soluble VEGF. Nonetheless, soluble VEGF induces significant effects in sites adjacent to tumors.

Regarding the Endothelial Progenitor Cells Project

The existence of a common endothelial/hematopoietic progenitor in the adult has been less clear. Nonetheless, several groups have reported that adult bone marrow can provide a reservoir of endothelial progenitor cell (EPCs) (reviewed in: Asahara 2000; Asahara & Isner 2002; Rabbany 2003). However, their potential to derive "blood cells" was not been explored.

EPCs have been detected in the peripheral blood and have the capacity to differentiate *in vivo* and *in vitro* (Asahara 1997; Shi 1998; Gehling 2000; Lin 2000; Reyes 2002). Their incorporation into sites of neovascularization has been also documented. Specifically, bone-marrow-derived EPCs have been shown to participate in neoangiogenesis after post-myocardial ischemia (Orlic 2001a, b & c; Kawamoto 2001 2003; Kocher 2001; Jackson 2001), limb ischemia (Takahashi 1999; Kalka 2000; Schatterman 2000; Iwaguro 2002; Yamaguchi 2003), wound healing (Asahara 1997, 1999a & b; Crosby 2000), atherosclerosis (Sata 2002), endothelialization of vascular grafts (Shi 1998; Bhattacharya 2000), retinal neovascularization (Otani 2002; Grant 2002), and cardiac impaired neovascularization during aging (Edelberg 2002).

Furthermore, the growth of at least certain tumors appears to be dependent on the recruitment of endothelial progenitor cells from bone marrow. Most significantly, transplantation of wild-type bone marrow into *Id3/4* knock-out mouse rescues the otherwise impaired tumor-neovascularization of *Id3/4* deficient animals. At least in this model system, tumor vascularization appears to depend entirely on bone marrow-derived cells (Lyden 2001; Rafii 2002). Thus, there is experimental evidence for the presence of circulating progenitor cells and there has been a plethora of studies directed towards testing the potential of these cells for therapeutic applications. Nonetheless much is yet to be learned about endothelial progenitor cells. What is the relative contribution of endothelial progenitor cells to sites of neovascularization in the adult (i.e., % of engraftment versus proliferation of local endothelial cells)? This question has been a point of heated debate, mostly because the assessments are not equal. Engraftment of endothelial progenitor cells, either from blood circulation (Asahara

1997; Murohara 2000, Lin 2000) or adult tissues (Jackson 2001) have been reported to range from 3.5 (Jackson 2001) to 10% (Crosby 2000) respectively. The information contrasts other studies that indicate progenitor contribution of up to 95% (Lyden 2001). Again, the source of bone-marrow (or purified subpopulation), and the experimental models used, have been variable. Therefore this question remains unexplored.

Another central question in the biology of EPCs is: What is their make-up? The marker (expression profile) of these so-called bone marrow-derived circulating endothelial progenitor cells is still unclear and the subject of active investigation. Many of the experiments have been performed using bone-marrow transplantation of ROSA26 donor mice into wild-type recipients. The ROSA26 mouse strain shows ubiquitous expression of betageo reporter gene and has been extremely useful to track cells derived from bone marrow (Zambrowicz 1997). Albeit useful in proving the principle that neo-endothelial cells are derived from transplanted marrow, these experiments do not offer information on cell surface or other markers (CD pattern) expressed by this marrow population. Experiments using CD34+ sorted precursors have shown that endothelial progenitor cells (EPCs) display this marker, both while in the bone marrow (Bhattacharya 2000) and also in the circulation (Asahara 1997). In contrast, other studies have reported the presence of tissue-resident CD34 negative cells that can contribute to muscle, as well as, endothelial cells (Goodell 1997; Majka 2003). These latter studies were performed with local progenitors, so it is possible that there are indeed multiple precursors, some bone-marrow-derived; some resident. More consistently CD34+, AC133 (CD133 or prominin A in mouse) and VEGFR2 are the markers most agreed upon in the literature (Peichev 2000; Salven 2003).

Why has it been difficult to fully characterize this population of bone marrow-derived cells? Models that can identify these cells are currently missing and the isolation of endothelial progenitor cells using CD34 is challenging, with low yield. In addition, CD34+ cells also include other progenitors. Therefore this population, while enriched, does not represent a highly pure population of endothelial progenitor cells. Purity is assessed by integration into the vascular wall, once they become incorporated the differentiation pathways are initiated and the make-up of the precursor population is lost. Consequently there is a strong need for genetically labeled model systems that would be less impacted by changes in differentiation programs. I am interested in pursuing this question as an expansion of this fellowship.

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Appendices

None